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Patentanmeldung Nr. Patent application No. Demande de brevet n°

01204785.8

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

Anmeldung Nr:
Application no.: 01204785.8
Demande no:

Anmeldetag:
Date of filing: 07.12.01
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Self-containing lactococcus strain

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C12N1/00

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of filing/Etats contractants désignées lors du dépôt:

AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE TR

SELF-CONTAINING *Lactococcus* STRAIN

The invention relates to a recombinant *Lactococcus* strain, with environmentally limited growth and viability. More particularly, it relates to a recombinant *Lactococcus* that can only survive in a medium, where well-defined medium compounds are present. A preferred embodiment is a *Lactococcus* that may only survive in a host organism, where said medium compounds are present, but cannot survive outside the host organism in absence of said medium compounds.

Lactic acid bacteria have long time been used in a wide variety of industrial fermentation processes. They have generally-regarded-as-safe status, making them potentially useful organisms for the production of commercially important proteins. Indeed, several heterologous proteins, such as Interleukin-2, have been successfully produced in *Lactococcus* spp (Steidler *et al.*, 1995). It is, however, unwanted that such genetically modified microorganisms are surviving and spreading in the environment.

To avoid unintentional release of genetically modified microorganisms, special guidelines for safe handling and technical requirements for physical containment are used. Although this may be useful in industrial fermentations, the physical containment is generally not considered as sufficient, and additional biological containment measures are taken to reduce the possibility of survival of the genetically modified microorganism in the environment. Biological containment is extremely important in cases where physical containment is is difficult or even not applicable. This is, amongst others, the case in applications where genetically modified microorganisms are used as live vaccines or as vehicle for delivery of therapeutical compounds. Such applications have been described e.g. in WO 97/14806, which discloses the delivery of biologically active peptides, such as cytokines, to a subject, by recombinant non-invasive or non-pathogenic bacteria. WO 96/11277 describes the delivery of therapeutic compounds to an animal – including humans - by administration of a recombinant bacterium, encoding the therapeutic protein. Steidler *et al.* (2000) describe the treatment of colitis by administration of a recombinant *Lactococcus lactis*, secreting interleukin-10. Such a delivery may indeed be extremely useful to treat a disease in an affected human or animal, but the recombinant bacterium may act as a harmful and pathogenic microorganism when it enters a non-

affected subject, and an efficient biological containment that avoids such unintentional spreading of the microorganism is needed.

Biological containment systems for host organisms may be passive, based on a strict requirement of the host for specific growth factor or a nutrient, that is not present or present in low concentrations in the outside environment, or active, based on so-called suicidal genetic elements in the host, whereby the host is killed in the outside environment by a cell killing function, encoded by a gene that is under control of a promoter only being expressed under specific environmental conditions.

Passive biological containment systems are well known in mircoorganisms such as *Escherichia coli* or *Saccharomyces cerevisiae*. Such *E. coli* strains are disclosed e.g. in US4100495. WO 95/1061 discloses lactic acid bacterial suppressor mutants and their use as means of containment in lactic acid bacteria, but in that case, the containment is on the level of the plasmid, rather than on the level of the host strain and it stabilizes the plasmid in the host strain, but doesn't provide containment for the genetically modified host strain itself.

Active suicidal systems have been described by several authors. Such system consists of two elements: a lethal gene, and a control sequence that switches on the expression of the lethal gene under non-permissive conditions. WO 95/10614 discloses the use of a cytoplasmatically active truncated and/or mutated *Staphylococcus aureus* nuclease as lethal gene. WO 96/40947 discloses a recombinant bacterial system with environmentally limited viability, based on the expression of either an essential gene, expressed when the cell is in the permissive environment and is not expressed or temporarily expressed when the cell is in the non-permissive environment and/or a lethal gene, wherein expression of the gene is lethal to the cell and the lethal gene is expressed when the cell is in the non-permissive environment but not when the cell is in the permissive environment. WO 99/58652 describes a biological containment system based on the relE cytotoxin. However, most systems have been elaborated for *Escherichia coli* (Tedkin *et al.*, 1995; Knudsen *et al.*, 1995; Schweder *et al.*, 1995) or for *Pseudomonas* (Kaplan *et al.*, 1999; Molino *et al.*, 1998). Although several of the containment systems theoretically can by applied to lactic acid bacteria, no specific biological containment systems for *Lactococcus* have been described.

It is the objective of the present invention to provide a suitable biological containment system for *Lactococcus*.

A first aspect of the invention is an isolated strain of *Lactococcus* sp. comprising a defective thymidylate synthase gene. Preferably, said defective thymidylate synthase gene is inactivated by gene disruption. Even more preferably, said *Lactococcus* sp. is *Lactococcus lactis*. A special embodiment is a *Lactococcus* sp. strain, preferably *Lactococcus lactis*, more preferably a *Lactococcus lactis* MG1363 derivative, whereby the thymidylate synthase gene has been disrupted and replaced by and replaced by a human interleukin-10 expression unit.

Another aspect of the invention is the use of a strain according to the invention as host strain for transformation, whereby the transforming plasmid does not comprise an intact thymidylate synthase gene.

Still another aspect of the invention is a transformed strain of *Lactococcus* sp. according to the invention, comprising a plasmid that does not comprise an intact thymidylate synthase gene.

Another aspect of the invention is a medical preparation, comprising a transformed strain of *Lactococcus* sp., according to the invention.

The *Lactococcus lactis* subsp. *lactis* thymidylate synthase gene (*thyA*) has been cloned by Ross *et al.* (1990a); it sequence is comprised in SEQ ID N° 3 and SEQ ID N° 5. EP0406003 discloses a vector devoid of antibiotic resistance and bearing a thymidylate synthase gene as a selection marker; the same vector has been described by Ross *et al.* (1990b). However, although it would have been logical to use this vector in a *Lactococcus lactis* strain, this has not been realized due to the lack of a suitable *thyA* mutant. Indeed, such a mutant has never been described. Surprisingly, we were able to construct such mutant by gene disruption, using homologous recombination in *Lactococcus*. In a preferred embodiment, the *thyA* gene is disrupted by a functional human interleukin-10 expression cassette. However, it is clear that any construct can be used for gene disruption, as long as it results in an inactivation of the *thyA* gene or in an inactive thymidylate synthase. As a non-limiting example, the homologous recombination may result in a deletion of the gene, in one or more amino acid substitutions that lead to an inactive form of the thymidylate synthase, or to a frameshift mutation resulting in a truncated form of the protein.

Such a *Lactococcus* sp. *thyA* mutant is very useful as a host strain for transformation, in situations where more severe containment than purely physical containment is needed. Indeed, it is known that *thyA* mutants cannot survive in an environment without, or with only a limited concentration of thymidine and/or thymine. When such a

strain is transformed with a plasmid that doesn't comprise an intact *thyA* gene and cannot complement the mutation, the transformed strain will become suicidal in a thymidine/thymine poor environment. Such a strain can be used in a fermentor, as an additional protection for the physical containment, but is especially useful in cases where the strain is used as a delivery vehicle in an animal body. Indeed, when such a transformed strain is given orally to an animal – including humans – it will survive in the gut, provided a sufficiently high concentration of thymidine/thymine is present, and will produce homologous and/or heterologous proteins that may be beneficial for said animal. However, once said strain is secreted in the environment, e.g. in the faeces, it will not be able to survive any longer.

The transforming plasmid can be any plasmid, as long as it cannot complement the *thyA* mutation. It may be a selfreplicating plasmid that preferably carries one or more genes of interest and one or more resistance markers, or it may be an integrative plasmid. In the latter case, the integrative plasmid itself may be used to create the mutation, by causing integration at the *thyA* site, whereby the *thyA* gene is inactivated. Preferably, the active *thyA* gene is replaced by double homologous recombination by a cassette comprising the gene or genes of interest, flanked by targetting sequences that target the insertion to the *thyA* target site. It is of extreme importance that these sequences are sufficiently long and sufficiently homologous to obtain to integrate the sequence into the target site. Preferably, said targeting sequences consist of at least 100 contiguous nucleotides of SEQ ID N°1 at one side of the gene of interest, and at least 100 contiguous nucleotides of SEQ ID N°2 at the other side; more preferably, said targeting sequences consists of at least 500 contiguous nucleotides of SEQ ID N°1 at one side of the gene of interest, and at least 500 contiguous nucleotides of the SEQ ID N° 2 at the other side; most preferably, said targeting sequences consists of SEQ ID N°1 at one side of the gene of interest and SEQ ID N°2 at the other side, or said targeting sequences consist of at least 100 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID N° 1 at one side of the gene of interest, and of at least 100 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID N° 2 at the other side of the gene of interest, preferably said targeting sequences consist of at least 500 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID N° 1 at one side of the gene of interest, and of at least 500 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID N° 2 at the other side of the gene of

interest, most preferably said targeting sequences consist of at least 1000 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID N° 1 at one side of the gene of interest, and of at least 1000 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID N° 2 at the other side of the gene of interest . The percentage identity is measured with BLAST, according to Altschul *et al.* (1997). A preferred example of a sequence, homologous to SEQ ID N°1 is given in SEQ ID N° 7. For the purpose of the invention, SEQ ID N° 1 and SEQ ID N° 7 are interchangeable.

Transformation methods of *Lactococcus* are known to the person skilled in the art, and include, but are not limited to protoplast transformation and electroporation.

A transformed *Lactococcus* sp. strain according to the invention is useful for the delivery of prophylactic and/or therapeutical molecules and can be used in a pharmaceutical composition. The delivery of such molecules has been disclosed, as a non-limiting example, in WO 97/14806 and in WO 98/31786. Prophylactic and/or therapeutical molecules include, but are not limited to polypetides such as insuline, growth hormone, prolactine, calcitonin, group 1 cytokines, group 2 cytokines and group 3 cytokines and polysaccharides such as polysaccharide antigens from pathogenic bacteria. A preferred embodiment is the use of a *Lactococcus* sp. strain according to the invention to deliver human interleukin-10. This strain can be used in the manufacture of a medicament to treat Crohn's disease.

Brief description of the figures

Figure 1: Map of the MG1363 *thyA* locus

Figure 2: Schematic representation of *thy*A loci of genetically engineered *thy*A negative *L. lactis* strains containing different hIL-10 expression units. Black parts represent original *L. lactis* MG1363 genetic information, white parts represent recombinant genetic information.

Figure 3: PCR identification of Thy11 (Thy11 1.1 and Thy11 7.1 represent individually obtained, identical clones). Standard PCR reactions were performed by using aliquots of saturated cultures of the indicated strains as a source of DNA template. Panel A shows an agarose gel of the products of the indicated PCR reactions. Panel B shows the positions at which primers attach in the thyA (1), upstream (2) or downstream (3) PCR's. Oligonucleotide primers used: (1): ATgACTTACgCAgATCAAgTTTTT and

TTAAATTgCTAAATCAAATTTCAATTg (2): TCTgATTgAgTACCTTgACC and gCAATCATAATTggTTTTATTg (3): CTTACATgACTATgAAAATCCg and cTTTTTTATTATTAgggAAAgCA

Figure 4: Southern blot analysis of the indicated strains. Chromosomal DNA was extracted and digested with the indicated restriction enzymes. Following agarose gel electrophoresis the DNA was transferred to a membrane and the chromosome structure around the thyA locus was revealed by use of DIG labelled thyA or hIL-10 DNA fragments (panel A). Panel B shows a schematic overview of the predicted structure of the thyA locus in both MG1363 and Thy11.

Figure 5: Production of hIL-10. Panel A shows a western blot revealed with anti-hIL-10 antiserum of culture supernatant and cell associated proteins of the indicated strains. Panel B shows quantification (by ELISA) of hIL-10 present in the culture supernatant.

Figure 6: Growth rate of the indicated strains in GM17 containing 100µg/ml (T100) 50µg/ml (T50) 25µg/ml (T25) or no (T0) extra thymidine and possibly supplemented with 5µg/ml of erythromycin (E). Saturated overnight cultures (prepared in T50) were diluted 1:100 in the indicated culture media. Panel A shows the kinetics of absorbance accumulation. Panel B shows the kinetics of the number of colony forming units (cfu) per ml of culture.

Examples

From L. lactis MG1363 (Gasson, 1983) we have cloned out the regions flanking the sequence according to Ross *et al.* (1990a)

The knowledge of these sequences is of critical importance for the genetic engineering of any lactococcus strain in a way as described below, as the strategy will employ double homologous recombination in the areas 1000 bp at the 5'end (SEQ ID N°1) and 1000 bp at the 3'end (SEQ ID N°2) of thyA, the "thyA target". These sequences are not available from any public source to date. We have cloned these flanking DNA fragments and have identified their sequence. The sequence of the whole locus is shown in SEQ ID N°3; a mutant version of this sequence is shown in SEQ ID N°5. Both the 5' and 3' sequences are different from the sequence at genbank AE006385 describing the *L. lactis* IL1403 sequence (Bolotin, in press) or at AF336368 describing the *L. lactis* subsp. *lactis* CHCC373 sequence. From the literature it is obvious that homologous recombination by use of the published

sequences adjacent to *thyA* (Ross *et al.*, 1990a) (86 bp at the 5'end and 31 bp at the 3'end) is virtually impossible due to the shortness of the sequences. Indeed, Biswas *et al.* (1993) describe a logarithmically decreasing correlation between length of the homologous sequences and frequency of integration.

The *thyA* replacement is performed by making suitable replacements in a plasmid borne version of the *thyA* target, as described below. The carrier plasmid is a derivative of pORI19 (Law *et al.*, 1995) a replication defective plasmid, which only transfers the erythromycin resistance to a given strain when a first homologous recombination, at either the 5' 1000bp or at the 3'1000bp of the *thyA* target. A second homologous recombination at the 3' 1000bp or at the 5' 1000bp of the *thyA* target yields the desired strain.

The *thyA* gene is replaced by a synthetic gene encoding a protein which has the *L. lactis* Usp45 secretion leader (van Asseldonk *et al.*, 1990) fused to a protein of identical amino acid sequence than: (a) the mature part of human-interleukin 10 (hIL-10) or (b) the mature part of hIL-10 in which proline at position 2 had been replaced with alanine or (c) the mature part of hIL-10 in which the first two amino acids had been deleted; (a), (b) and (c) are called hIL-10 analogs, the fusion products are called Usp45-hIL-10.

The *thyA* gene is replaced by an expression unit comprising the lactococcal P1 promotor (Waterfield *et al.*, 1995), the *E. coli* bacteriophageT7 expression signals: putative RNA stabilising sequence and modified gene10 ribosomal binding site (Wells and Schofield, 1996).

At the 5' end the insertion is performed in such way that the ATG of thyA is fused to the P1-T7Usp45-hIL-10 expression unit.

```
5'agataggaaaatttcatgacttacgcagatcaagttttt...thyA wild type
                    gattaagtcatcttacctctt...P1-T7-usp45-hIL10
5'agataggaaaatttcatggattaagtcatcttacctctt...thyA-,P1-T7-usp45-
hIL10
```

Alternatively, at the 5' end the insertion is performed in such way that the thyA ATG is not included:

```
5'agataggaaaatttcacttacgcagatcaagttttt...thyA wild type
                  gattaagtcatcttacctctt...P1-T7-usp45-hIL10
```

5'agataggaaaatttcgattaagtcatcttacctctt...thyA⁻,P1-T7-usp45-hIL10

Alternatively, at the 5' end the insertion is performed in such way that the thyA promotor [Ross, 1990 a] is not included:

5'tctgagaggttattttgggaaatactattgaaccatatcgaggtgtgtggtataatgaaggg
gaattaaaaaagataggaaaatttcatg...thyA wild type

gattaagtcatcttacctctt...P1-T7-usp45-hIL10

5'tctgagaggttattttgggaaatactagattaagtcatcttacctctt...thyA⁻,P1-T7-usp45-hIL10

At the 3' end an ACTAGT SpeI restriction site was engineered immediately adjacent to the TAA stop codon of the usp45-hIL-10 sequence. This was ligated in a TCTAGA *Xba*I restriction site, which was engineered immediately following the thyA stop codon

aaaatccgtaactaactagt3'...usp45-hIL10
gatttagcaatttaaattaaattaatctataagtt3'...thyA-wild type
tctagaattaatctataagttactga3'...engineered thyA target
aaaatccgtaactaactagaattaatctataagttactga3'...thyA⁻,usp45-hIL10

These constructs are depicted in figure 2

The resulting strains are *thyA* deficient, a mutant not yet described for *L. lactis*. It is strictly dependent upon the addition of thymine or thymidine for growth.

The map of the deletion, as well as the PCR analysis of two isolates of a representative mutant is shown in figure 3. The presence of the thymidylate synthase and the interleukin 10 gene in the wild type strain and in those two independent isolates of the mutant was analyzed by Southern analysis shown in figure 4.

Human interleukin 10 production in the mutants was checked by western blot analysis, and compared with the parental strain, transformed with pTREX1 as negative control, and the parental strain, transformed with the IL10 producing plasmid pT1HIL10apxa as positive control (figure 5A). The concentration in the culture supernatant was quantified using ELISA. As shown in figure 5B, both isolates of the mutant produce a comparable, significant amount of hIL-10, be it far less than the strain, transformed with the non intergrative plasmid pT1HIL10apxa.

The effect of the thymidilate synthase deletion on the growth in thymidine less and thymidine supplemented media was tested; the results are summarized in figure 6. Absence of thymidine in the medium strongly limits the growth of the mutant, and even results in a decrease of colony forming units after four hours of cultivation. Addition of thymidine to the medium results in an identical growth curve and amount of colony forming units, compared to the wild type strain, indicating that the mutant doesn't affect the growth or viability in thymidine supplemented medium

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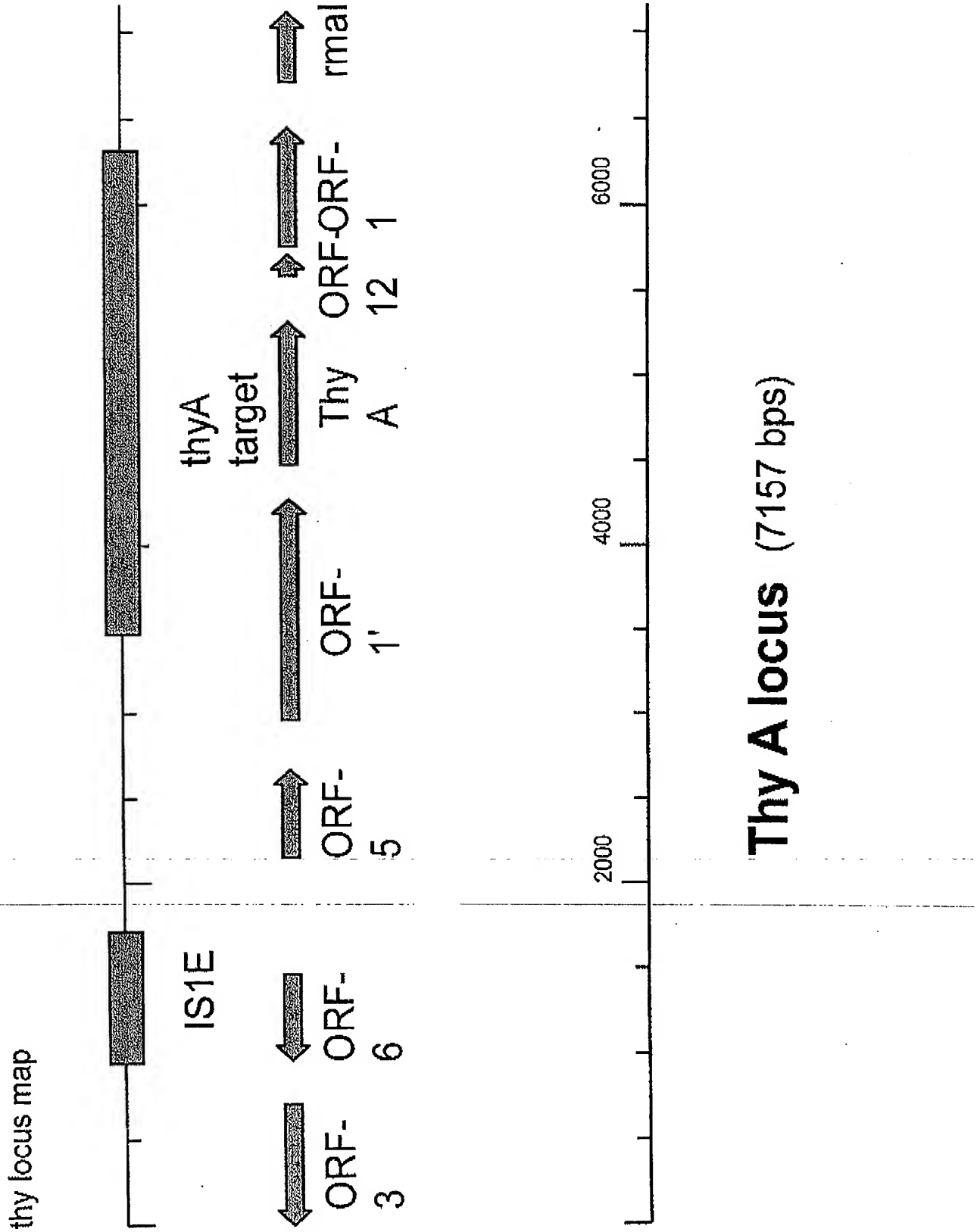
Claims

1. An isolated strain of *Lactococcus* sp. comprising a defective thymidylate synthase gene.
2. A strain of *Lactococcus* sp. according to claim 1, whereby said gene is inactivated by gene disruption.
3. An isolated strain of *Lactococcus* sp. according to claim 1 or 2, whereby said *Lactococcus* sp. is *Lactococcus lactis.*
4. The use of a strain of *Lactococcus* sp. according to any of the claims 1-3 as host strain for transformation, whereby the transforming plasmid does not comprise an intact thymidylate synthase gene.
5. A transformed strain of *Lactococcus* sp. according to any of the claims 1-3, comprising a transforming plasmid that does not comprise an intact thymidylate synthase gene.
6. A pharmaceutical composition comprising a transformed strain of *Lactococcus* sp. according to claim 5

Abstract

The invention relates to a recombinant *Lactococcus* strain, with environmentally limited growth and viability. More particularly, it relates to a recombinant *Lactococcus* that can only survive in a medium, where well-defined medium compounds are present. A preferred embodiment is a *Lactococcus* that may only survive in a host organism, where said medium compounds are present, but cannot survive outside the host organism in absence of said medium compounds.

Figure 1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IS1E
ORF-3
ORF-6
ORF-5
ORF-1'
thyA target
Thy A
ORF-1
ORF-2
rmal
2000
4000
6000
Thy A locus (7157 bps)

Figure 2

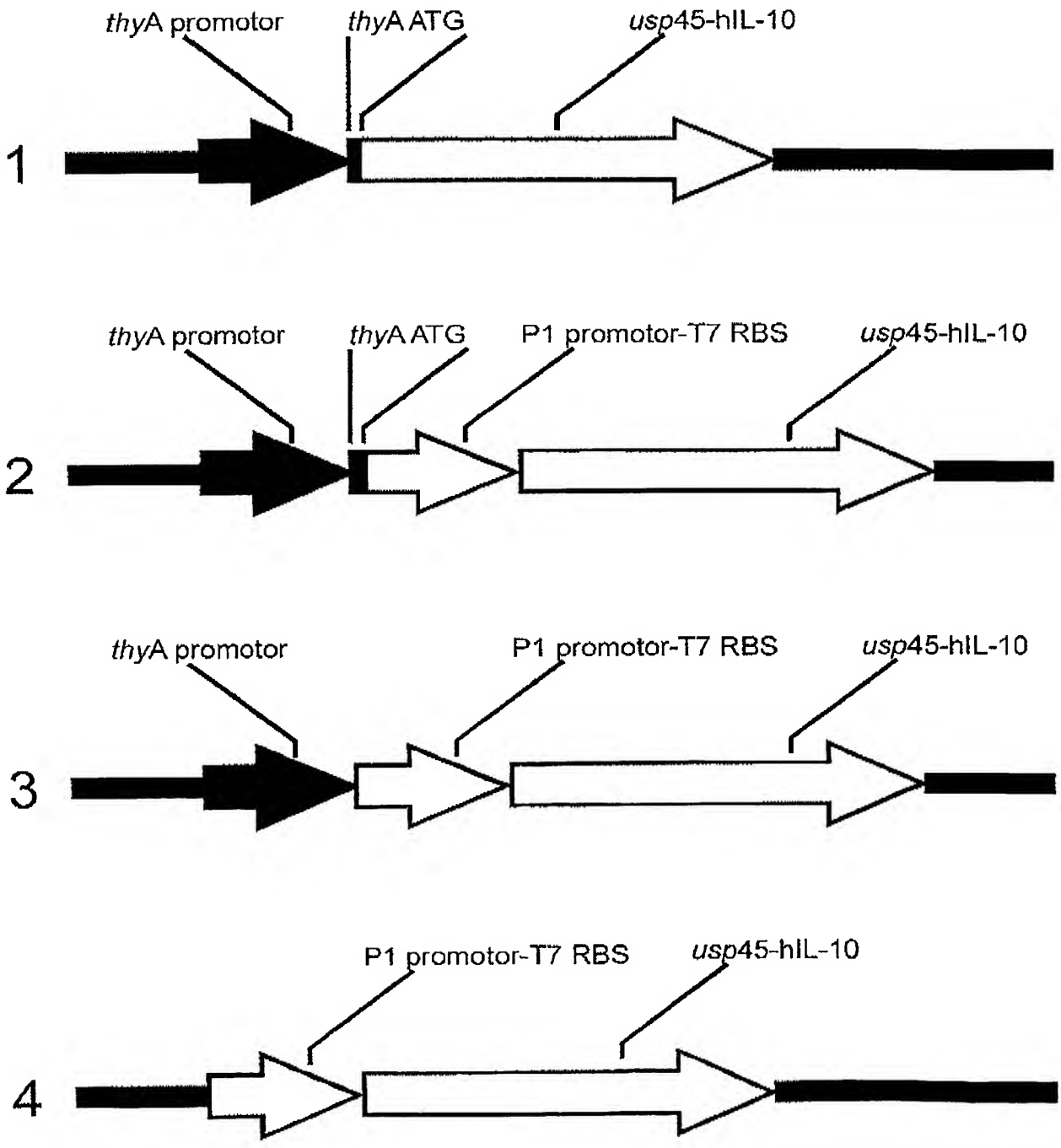
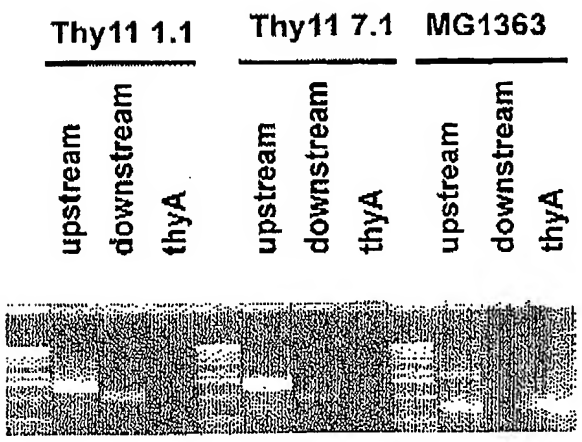

thyA promotor
thyA ATG
*usp*45-hIL-10
1
thyA promotor
thyA ATG
P1 promotor-T7 RBS
*usp*45-hIL-10
2
thyA promotor
P1 promotor-T7 RBS
*usp*45-hIL-10
3
P1 promotor-T7 RBS
*usp*45-hIL-10
4

Figure 3

A


Thy11 1.1
Thy11 7.1
MG1363
upstream
downstream
thyA
upstream
downstream
thyA
upstream
downstream
thyA

B

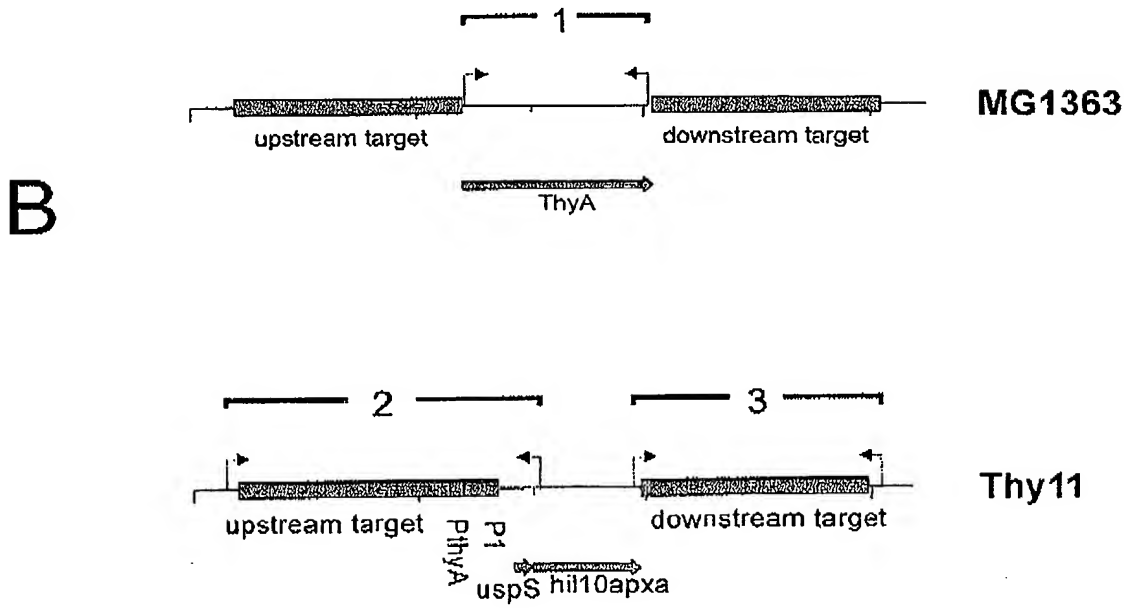
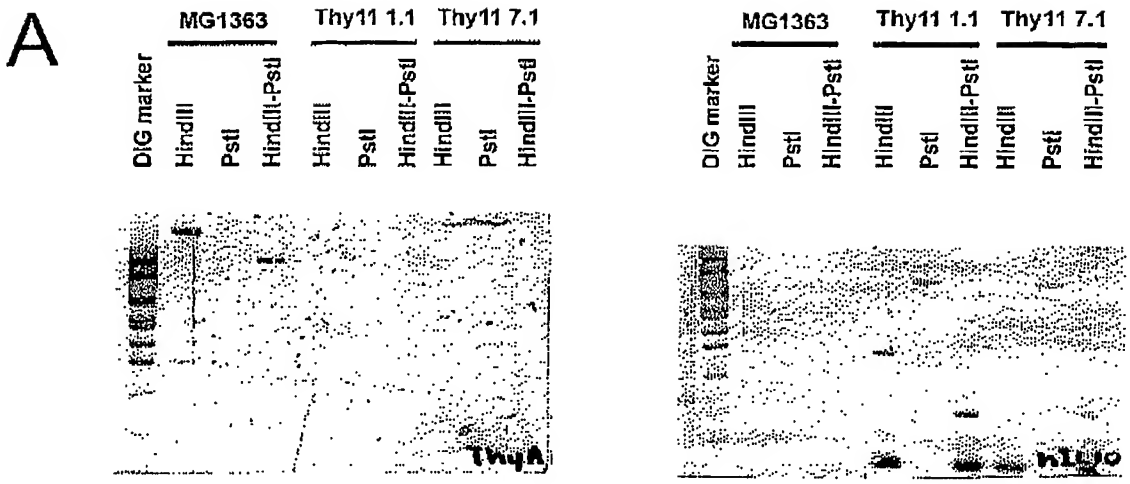

1
MG1363
upstream target
downstream target
ThyA
2
3
Thy11
upstream target
PthyA
P1
downstream target
uspS hil10apxa
1000
2000
3000

Figure 4



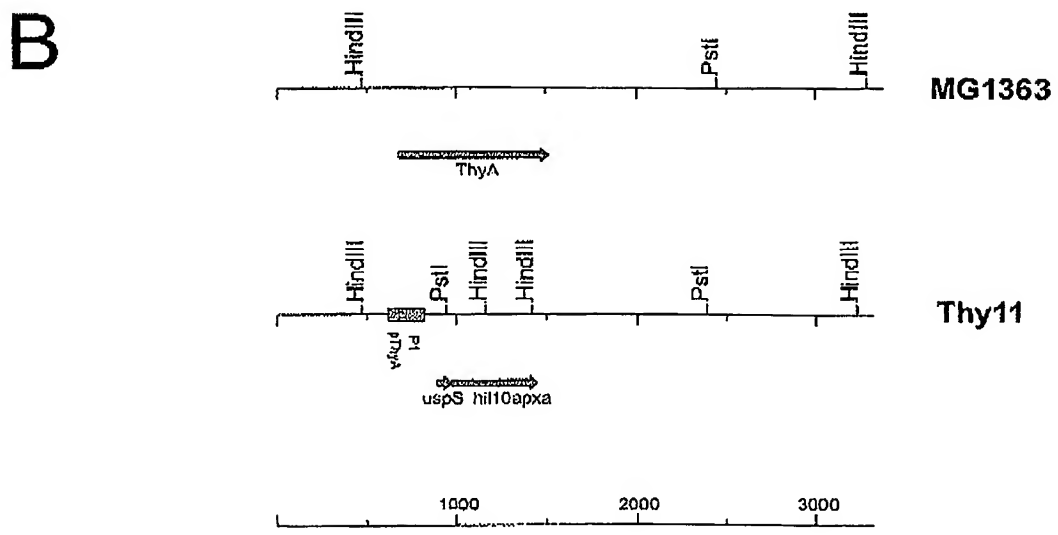
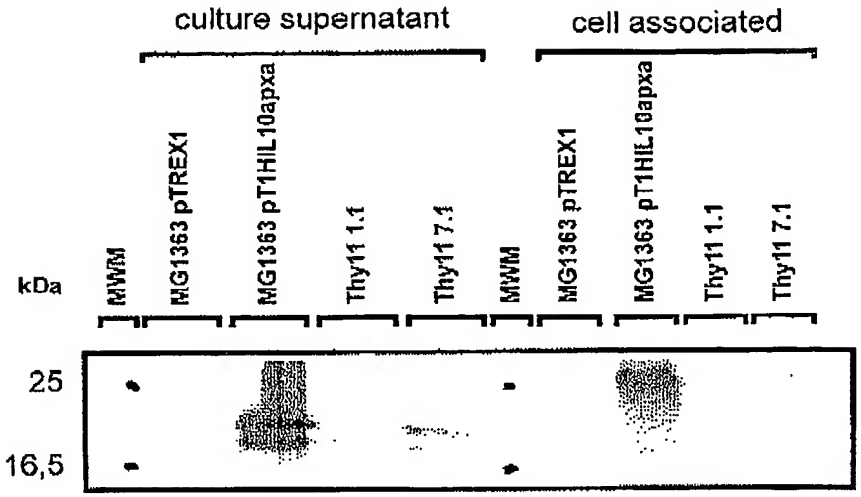


Figure 5

A



B

	hIL10PxA		pTREX1		Thy11 1.1		Thy11 7.1	
	sample 1	sample 2	sample 3	sample 4	sample 5	sample 6	sample 7	sample 8
concentration (ng/ml)	131,34	123,01	0	0	2,55	1,8	2,8	2,72
std (ng/ml)	0	15,27	0	0	1,05	0	0,95	0,53
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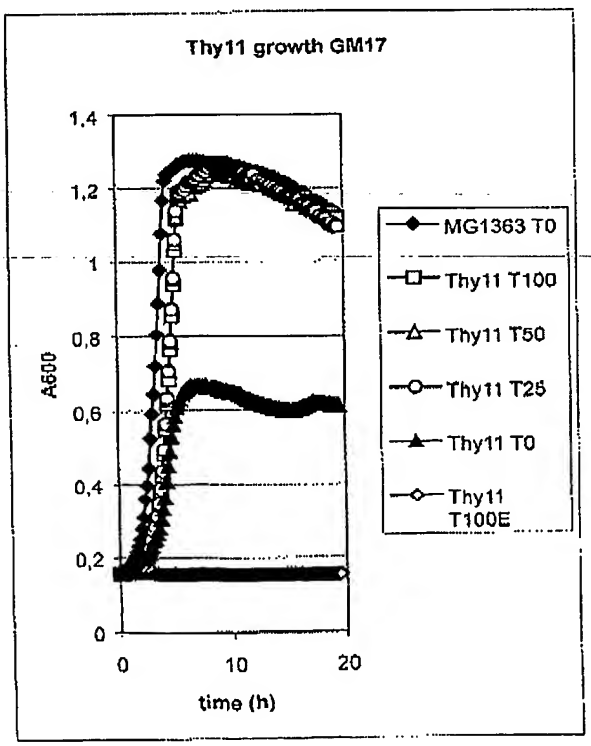
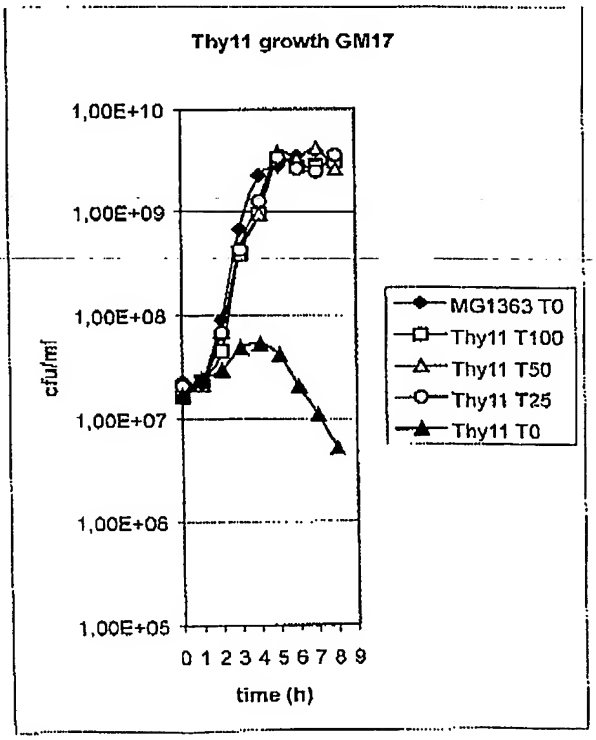
Figure 6





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Asn Leu Trp Gln Tyr Glu Asp Phe Glu Glu Thr Glu Gly Leu Leu Pro
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Cys Ala Phe Gln Thr Met Phe Asp Val Arg Arg Glu Lys Asp Gly Gln
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Ala His His Ile Asn Ala Met Gln Tyr Val Ala Leu Gln Met Met Ile
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Ala Lys His Phe Ser Trp Lys Val Gly Lys Phe Phe Tyr Phe Val Asn
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aat tta cat att tat gat aat cag ttt gag cag gca aat gaa tta atg      5164
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Lys Arg Thr Ala Ser Glu Lys Glu Pro Arg Leu Val Leu Asn Val Pro
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Asp Gly Thr Asn Phe Phe Asp Ile Lys Pro Glu Asp Phe Glu Leu Val
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Asp Tyr Glu Pro Val Lys Pro Gln Leu Lys Phe Asp Leu Ala Ile
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Glu Leu Ser Val Leu Glu Glu Lys Tyr Gly Val Lys Tyr Trp Gly Glu
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Lys Lys Tyr Asn Ile Ile Gly Lys Leu Leu Glu Gly Leu Ala Lys Asn
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Pro Trp Asn Arg Arg Asn Ile Ile Asn Leu Trp Gln Tyr Glu Asp Phe
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Glu Glu Thr Glu Gly Leu Leu Pro Cys Ala Phe Gln Thr Met Phe Asp
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Gln Arg Ser Asn Asp Met Leu Val Ala His His Ile Asn Ala Met Gln
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Tyr Val Ala Leu Gln Met Met Ile Ala Lys His Phe Ser Trp Lys Val
195 200 205

Gly Lys Phe Phe Tyr Phe Val Asn Asn Leu His Ile Tyr Asp Asn Gln
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Phe Glu Gln Ala Asn Glu Leu Met Lys Arg Thr Ala Ser Glu Lys Glu
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